Hyperketonemia during LPS induced mastitis affects systemic andl local intramammary metabolism in dairy cows

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Interpretive summary**: Hyperketonemia during LPS induced mastitis affects systemic and local intramammary metabolism in dairy cows.** *By Zarrin et al*. Effects of an induced hyperketonemia during an intramammary lipopolysaccharide (LPS) challenge on systemic and local mammary metabolism in mid-lactation dairy cows demonstrated that hyperketonemia had no effect on mammary metabolism. Induced intramammary LPS mastitis increased plasma glucose, cortisol, glucagon, insulin concentration, and decreased plasma beta-hydroxybutyrate (BHBA) concentration while plasma glucose and glucagon increased less in the BHBA treated animals than in controls. The results indicate that BHBA infusion decreases glucose as an energy source for the immune system through the decline of glucagon which may reflect the negative effect of spontaneous hyperketonemia on metabolic adaptations during mastitis in dairy cows.

HYPERKETONEMIA AND MAMMARY LPS CHALLENGE

Hyperketonemia during LPS induced mastitis affects systemic and local intramammary metabolism in dairy cows

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ABSTRACT

Hyperketonemia interferes with the metabolic regulation in dairy cows. It is assumed that metabolic and endocrine changes during hyperketonemia affect also metabolic adaptations during inflammatory processes. We have therefore studied systemic and local intramammary effects of elevated plasma beta-hydroxybutyrate (BHBA) before and during the response to an intramammary lipopolysaccharide (LPS) challenge. Thirteen dairy cows received intravenously either a Na-DL-β-OH-butyrate infusion (HyperB, n=5) to achieve a constant plasma BHBA concentration (1.7 ± 0.1 mmol/L), adjustments of the infusion rates were made based on immediate measurements of plasma BHBA every 15 min, or an infusion with a 0.9 % NaCl solution (Control, n=8) for 56 h. Infusions started at 0900 am on day 1 and continued until 0500 pm two days later. Two udder quarters were challenged with 200 µg *Escherichia coli*-LPS and two udder quarters were treated with 0.9 % saline solution as control quarters at 48 h after the start of infusion. Blood samples were taken at one week and 2 h before the start of infusions as reference samples and hourly during the infusion. Mammary gland biopsies were taken one week before, 48 h, and 56 h (8 h after LPS challenge) after the start of infusions. The mRNA abundance of key factors related to BHBA and fatty acid metabolism, and glucose transporters was determined in mammary tissue biopsies. Blood samples were analyzed for plasma glucose, BHBA, non-esterified fatty acid (NEFA), urea, insulin, glucagon, and cortisol concentration. Differences were not different for effects of BHBA infusion on the mRNA abundance of any of the measured target genes in the mammary gland before LPS challenge. Intramammary LPS challenge increased plasma glucose, cortisol, glucagon, and insulin concentration in both groups ($P<0.05$) but increases in plasma glucose and glucagon concentration were less pronounced in HyperB than in controls ($P<0.05$). In response to LPS challenge plasma BHBA concentration decreased in controls ($P<0.05$), and decreased also slightly in the BHBA infused animals because the BHBA concentration could

58 not be fully maintained despite a rapid increase of BHBA infusion rate ($P<0.001$). The
59 change of mRNA abundance of citrate synthase (CS) in LPS-quarters, was significant
60 between two treatment groups ($P<0.05$). Results indicate that elevated circulating BHBA
61 inhibits gluconeogenesis before and during immune response to LPS challenge likely because
62 BHBA can replace glucose as an energy source.

63

64 INTRODUCTION

65 The markedly increased energy and nutrient requirements at simultaneously inadequate feed
66 intake results in a negative energy balance (NEB) at the onset of lactation (van Dorland et al.,
67 2009; Gross et al., 2011). A severe NEB leads to an increased mobilization of fat stores, an
68 elevated uptake of NEFA by the liver, and an increase of plasma ketone body concentration
69 (Bobe et al., 2004; Gross et al., 2011). The concentration of plasma BHBA, which is the
70 major circulating ketone body in ruminants, is increased as a compensatory response to
71 handle the excessive NEFA release during the NEB. Beta-hydroxybutyrate functions as
72 energy source in many tissues, especially during NEB, and it seems also to be used for citrate
73 synthesis in the mammary gland (Bionaz and Loor, 2008). An elevation of plasma BHBA
74 concentration beyond the threshold of 1,200 µmol/L (Ospina et al., 2010) is generally
75 accepted to represent the diagnosis of a subclinical ketosis (Duffield et al., 2009). The
76 elevation of plasma ketone body concentration increases the risk of clinical ketosis, displaced
77 abomasum, metritis and subsequent decrease of milk production (Duffield et al., 2009).
78 Elevated plasma BHBA concentration had a positive correlation with the severity of *E. coli*
79 mastitis in an in vitro study (Kremer et al., 1993), and the risk of mastitis was much higher in
80 subclinically ketotic dairy cows (Oltenacu and Ekesbo, 1994). High elevation of plasma
81 BHBA concentration more than physiological range impairs metabolism in animals (Müller et
82 al., 1984; Schlumbohm and Harmeyer, 2004; Zarrin et al., 2013a). From the present study two

papers have been already published. Induced hyperketonemia was shown decrease two plasma glucose and glucagon concentrations while it did not affect milk yield, feed intake, insulin, NEFA, urea, cortisol, and enzymes involved in gluconeogenesis (Zarrin et al., 2013a). In addition, BHBA infusion caused increased acute phase protein mRNA abundance in the mammary gland but not in the liver. The LPS-related increase of somatic cell counts (SCC) was less pronounced whereas mRNA abundance of IL-8, IL-10 increased more in the response to LPS in the group receiving BHBA infusion than the control animals (Zarrin et al., 2013b).

The use of intramammary LPS challenge to simulate intramammary infection and to induce mastitis in dairy cows was established previously (Bruckmaier et al., 1993) to investigate the effect of mastitis on metabolism, immune responses, and performance in dairy cows. There is evidence that LPS challenge affects metabolism and mRNA abundance of inflammatory and other factors (Waldron et al., 2006; Bruckmaier et al., 1993; Vernay et al., 2012). In addition, it causes a decline of milk production (Bannerman et al., 2003). However, there is no information about effects of induced hyperketonemia at simultaneously even or positive energy balance on metabolic changes during mastitis.

The objective was to induce long term hyperketonemia beyond the threshold (1.2 mmol/L) through BHBA infusion for 56 h, and additionally stimulate the immune system by intramammary LPS challenge to investigate systemic metabolic effects and changes of the mRNA abundance of genes related to metabolism in mammary tissue before and during the LPS challenge in mid-lactating dairy cows. Genes involved in fatty acid synthesis (fatty acid synthase [FASN], acetyl-CoA carboxylase [ACoAC]), tricarboxylic acid cycle (CS), mammary BHBA metabolism (beta-hydroxybutyrate dehydrogenase 1 [BDH1], beta-hydroxybutyrate dehydrogenase 2 [BDH2], Succinyl-CoA:3-ketoacid-coenzyme A transferase 1 [OXCT1]), and glucose transporters (glucose transporter 1 [GLUT1], glucose transporter 4

[GLUT4]) were selected to investigate the effects of elevated BHBA at simultaneously reduced glucose on the mammary metabolism.

MATERIAL AND METHODS

Animals and Management

The animal trials followed the Swiss Law on Animal Protection and were permitted by the Committee of Animal Experiments of the Canton Fribourg, Switzerland. In order to study the specific effect of BHBA infusion without the usual endocrine and metabolic changes during the transition period, thirteen multiparous Holstein dairy cows (parity 3.5 ± 0.1 Mean ± SEM) in a later lactational stage (28.0 ± 0.3 Mean ± SEM weeks of lactation) were selected. Before the start of experiments, health status of cows was checked by a routine blood glutaraldehyde coagulation test (Sandholm, 1976) and by measuring milk somatic cell count (DeLaval cell counter DCC, Delaval International AB, Tumba, Sweden), which had to be less than 150 x 10^3 cells/mL in all four quarters. Milking was performed twice daily at 0530 h and 1600 h.

Two weeks before the start of the experiment animals were kept in a tie stall barn for adaptation to the environment and feeding situations. All animals were fed ad libitum with hay (dry matter [DM] content, 890 g/kg of fresh matter [FM]; on DM basis, consisting of 153 g of crude protein [CP]/kg, 235 g of crude fiber [CF]/kg, and 5.7 MJ of NE_L/kg). In addition they received a protein- and energy-rich concentrate (23.5% barley, 14.0% oats, 20.0% wheat bran, 17.0% soybean expeller, 15.0% linseed meal, 0.6% salt, 2.2% carbonate of lime, 0.4% calf rearing feed premix, 4.0% molasses and 3.0% by-pass fat, DM content, 881 g/kg of FM; on DM basis, consisting of 217 g of CP/kg, 73.9 g of CF/kg, and 7.6 MJ of NE_L/kg) twice daily according to individual milk production. Cows had free access to fresh water and minerals (50 g/cow) were supplied daily.

133 ***Experimental design and treatments***

134 Thirteen dairy cows were randomly assigned to two treatment groups. One group was
135 intravenously infused with a pyrogen-free and pH-balanced Na-DL-β-OH-butyrate (HyperB,
136 n=5) to achieve an elevated plasma BHBA concentration (1.5 to 2.0 mmol/L) while the other
137 group received a 0.9 % saline solution (Control, n=8) for 56 h. The infusions started at 0900
138 am on day 1 and continued to 0500 pm two days later. Details on the preparation of solutions
139 and infusion procedures are explained elsewhere (Zarrin et al., 2013a).

140 At 48 h of infusions, two udder quarters were injected through the teat canal with 10 mL
141 physiological NaCl (0.9 %) include 200 µg of LPS *Escherichia coli* serotype 026:B6 (#
142 L8274; Sigma-Aldrich, St. Louis, MO) as LPS quarters, and 10 mL 0.9 % NaCl were injected
143 in two udder quarters as control. Details are described elsewhere (Vernay et al., 2012).

144

145 ***Data collection and sampling***

146 *Blood samples*. Blood samples were taken one week and 2 h before the start of infusions at
147 0730 h, after milking and before feeding, as reference samples and with sampling continuing
148 hourly during the entire infusion period. On day before the start of infusions cows were fitted
149 with indwelling intravenous catheters (Cavafix® Certo® Splittocan®, B. Braun Melsung AG,
150 Germany) with a length of 32 cm and a diameter of 16 G in both jugular veins. Blood samples
151 were collected into tubes, containing tri-potassium-EDTA, from the contralateral jugular
152 catheter, which was not used for the infusion. Samples were immediately put on wet ice,
153 centrifuged for 20 min at 3,000 x g, at +4 °C and plasma was stored at -20 °C until analysis.
154 During BHBA infusion, additional small blood samples (1mL) were taken and analyzed
155 immediately for adjustment of BHBA infusion rates every 5 min for the first 2 h and
156 thereafter every h.

157 *Udder biopsies.* Details of the udder biopsy procedure were described by Vernay et al. (2012).
158 In brief, one week before the start of infusions, before the LPS challenge (48 h after the start

of infusions), and at the end of infusion (56 h; 8 h after the LPS stimulation) udder tissue was taken from the two rear quarters (one LPS and one control quarter). Before the biopsies cows were sedated by an intravenous injection of 16 µg/kg of BW of xylazine (Xylazin Streuli ad us. vet.; G. Streuli & Co. AG, Uznach, Switzerland). Udder tissue (30 to 60 mg) was obtained under local anesthesia with 10 mL Lidocain 2% (Streuli Pharma AG, Uznach) using a biopsy needle (12 G x 10 cm; Bard® Magnum® Core Tissue Biopsy Needle, Türkenfeld, Germany). The samples were placed immediately into RNA stabilization reagent (RNAlater®, Ambion, Applied Biosysthems, Austin, TX), kept at +4 °C for 24 hours, and stored thereafter at –80 °C until RNA extraction.

Laboratory procedures

Blood variables. Plasma glucose, BHBA, NEFA, and urea concentrations were measured enzymatically with an automated analyzer (Cobas Mira 2, Hoffmann-La Roche, Basle, Switzerland) by commercial kits as described by van Dorland et al. (2009) and Kreipe et al. (2011). Plasma insulin was measured by radioimmunoassay (RIA) as described by Vicari et al. (2008), and total cortisol was measured by RIA as described by Blum et al. (1985). Plasma glucagon concentration was measured by using a commercial RIA kit (cat. # GL-32K, MILLIPORE, Zug, Switzerland). In addition the molar ratio of insulin: glucagon was calculated according the formula that suggested by Muller et al. (1971).

Udder tissue. Total RNA was extracted from udder tissues with peqGOLD TriFast™ (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Quantity and purity of RNA was measured by NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). Complementary DNA (cDNA) was synthesized by reverse transcription of one µg of total mRNA with Moleney Murine Leukemia Virus Reverse Transcriptase RNAase H Minus, Point Mutant (MMLV-RT; Promega Corp., Madison, WI) and random hexamer primers (Invitrogen, Leek, The Netherlands). The mRNA abundances of housekeeping genes

185 (glyceraldehyde 3-phosphate dehydrogenase [**GAPDH**] and **ubiquitin**) and target genes
186 related to udder metabolism were measured by real-time quantitative PCR (qPCR) in a Rotor-
187 Gene 6000 rotary analyzer (Corbett Research, Sydney, Australia) and software version 1.7.75.
188 The cycle threshold (CT) values obtained from candidate genes were adjusted according the
189 mean of the housekeeping genes CT according to the following equation: ΔCT = CT
190 (arithmetic mean of housekeeping genes) – CT (target gene). Differences in mRNA
191 abundance of candidate genes (based on CT values) before the start of infusions and 48 h after
192 the start of infusions were calculated: ΔΔCT = ΔCT [d 3 (0 h)] – ΔCT [d 0], and differences
193 in mRNA abundance by LPS challenge were calculated for LPS and control quarters
194 separately according to this equation: ΔΔCT = ΔCT [d 3 (8 h)] – ΔCT [d 3 (0 h)]. The primer
195 sequences for FASN, CS, ACoAC, BDH2, GLUT1, and GLUT4 were published elsewhere
196 (Graber et al., 2010). Succinyl-CoA:3-ketoacid-coenzyme A transferase 1 (OXCT1) and
197 BDH1 primers were designed to amplify cDNA. Selected Primer sequences of housekeeping
198 and measured genes encoding for mammary gland metabolism are shown in Table 1. The
199 means ± SEM of mRNA levels of GAPDH and ubiquitin were stable across time points
200 (baseline, 48 h after infusions, and at the end of infusions) and treatments (HyperB and NaCl)
201 are shown in Table 2.
202
203 ***Statistical analysis***
204 Before the statistical evaluation, all data tested for normality (SAS: proc univariate plot
205 normal), and all parameters followed a normal distribution. The changes (differences between
206 before and 48 h after the start of infusions, and before and after LPS administration) were
207 calculated for mRNA abundance of target genes. Area under the curve (AUC) was calculated
208 for blood variables during the LPS challenge (8.5 h) by the trapezoidal rule (combination of
209 rectangular and triangular area compartments). The respective plasma concentration before
210 the start of infusion was used as baseline value.

The changes of mRNA abundance of measured genes and AUC of blood variables were analyzed by using the general linear models (GLM) procedure of SAS (SAS Institute Inc., Cary, NC, USA, 2002-2008, Release 9.2), including treatment (BHBA or NaCl) as fixed effect. Differences between means were localized by Tukey's test. In addition, means of delta values obtained within each treatment were tested for their difference from "0". Differences in blood plasma variables between treatments within each time point and between time points within treatments were evaluated by using the MIXED procedure of SAS, including treatment (BHBA or NaCl), time, and their interaction as fixed effects, and cows as repeated subject. Tukey-Kramer test was used for adjustment of multiple comparisons. The Compound Symmetry (CS) structure was used for variance-covariance matrix for repeated measures within animals.

Data are presented as means ± SEM and differences were considered significant if $P<0.05$.

RESULTS

Infusion rates and concentration of plasma variables, feed intake, and milk yield

Area under the curve of plasma variables concentration in HyperB and control group during the LPS challenge (8.5 h) is shown in Table 4. Mean BHBA infusion rate and plasma BHBA concentration in HyperB and control group during -24 to -48 h of infusion and hourly after LPS challenge is shown in Figure 1. The mean infusion rate of BHBA over 48 h was 8.1 ± 0.3 µmol/kg/min. During 48 h BHBA infusion mean plasma BHBA concentration was maintained at 1.7 ± 0.1 mmol/L in HyperB group, and plasma BHBA concentration in the control group was 0.6 ± 0.1 mmol/L. Intramammary LPS challenge decreased plasma BHBA concentration in HyperB from 1.7± 0.1 to 1.4 ± 0.1 mmol/L ($P<0.001$) despite increase infusion rate. In control group plasma BHBA decreased from 0.6 ± 0.1 to 0.4 ± 0.1 mmol/L ($P<0.01$) compared with before LPS challenge. Because of adjustment BHBA infusion rate to maintain hyperketonemia in the HyperB group, mean BHBA infusion rate needed to be

237 significantly increased (P<0.001) to 11.1 µmol/kg/min after the LPS administration. Despite
238 the increased BHBA infusion rate in HyperB, plasma BHBA concentration decreased in both
239 groups (P<0.05) i.e. due to the fast change of BHBA plasma concentration the maintenance
240 of hyperketonemia in the HyperB through increased infusion rate was not completely
241 successful.
242 As previously reported (Zarrin et al., 2013a) dry matter intake (DMI) and milk yield were not
243 affected by 48 h BHBA infusion. Intramammary LPS challenge decreased DMI and milk
244 yield in both treatment groups, whereas these variables were not affected by treatments (Table
245 3).
246 Plasma glucose, glucagon, insulin, NEFA, urea, and cortisol concentration and molar ratio of
247 insulin: glucagon before the LPS challenge (average of 24-48 h of infusion), hourly after LPS
248 stimulation (8.5 h), and their differences within treatments and between each time points are
249 shown in Figures 2A, 2B, 2C, 3A, 3B, 3C, 4. Compared to plasma glucose concentration
250 before the LPS challenge, intramammary LPS administration caused an increase of plasma
251 glucose concentration in both infusion groups up to 90 min. Plasma glucose concentration
252 was decreased at 150 min after the LPS challenge, after that increased again at 210 min, and
253 finally decreased to the same concentration as before LPS administration. The increase of
254 plasma glucose concentration after LPS administration was lower in HyperB than in the
255 Control (P<0.05; Figure 2A; Table 4).
256 On the second day of hyperketonemia, treated cows had on average lower glucagon
257 concentrations than control cows. Intramammary LPS treatment increased after 3.5 h
258 glucagon concentration, which persisted for the remaining sampling period (8.5 h). During
259 this time period, glucagon concentrations were lower in hyperketotic than control cows
260 (P<0.05; Figure 2B), indicating that hyperketonemia attenuates the LPS-induced glucagon
261 secretion (P<0.05; Table 4).

In comparison with 48 h BHBA infusion, plasma insulin increased ($P<0.05$; Figure 2C) in response to LPS challenge and reached a peak at 210 min in HyperB and at 270 min in control group, respectively. Based on AUC plasma insulin concentration did not differ between HyperB and control group (Table 4).

Plasma NEFA concentration was not affected by the LPS challenge and BHBA infusion (Figure 3A; Table 4).

Intramammary LPS administration induced a decrease of plasma urea concentration only in the first 90 min after the LPS challenge in HyperB group, whereas LPS challenge decreased that in the first 30 min in control group ($P<0.05$; Figure 3B). Urea concentrations were not affected by treatments infusion (Table 4).

Plasma cortisol concentration was increased at 90 min after the intramammary LPS stimulation in both treatment groups ($P<0.05$; Figure 3C), but based on AUC cortisol did not differ between HyperB and control group (Table 4).

Insulin: glucagon molar ratio was not affected by the treatments at second day of infusion and during the LPS challenge, while it affected by intramammary LPS challenge at 3.5 h and 4.5 h in HyperB and control groups, respectively (Figure 4; Table 4).

mRNA abundance of candidate genes related to mammary gland metabolism

mRNA abundance related to mammary metabolism during the 48 h of BHBA infusion. The mRNA abundance of genes related to mammary gland metabolism before and 48 h after the start of infusions are shown in Table 4. Fatty acid synthase mRNA abundance decreased within the HyperB group during 48 h of BHBA infusion ($P<0.05$; Table 4). The mRNA abundances of the other candidate genes were not affected by 48 h BHBA infusion.

mRNA abundance of genes related to mammary metabolism during the LPS challenge. For both groups, differences between mRNA abundance of genes related to mammary gland metabolism before and after the LPS challenge in quarters stimulated with intramammary

LPS and control quarters are shown in Table 4. Citrate synthase mRNA abundance in LPS quarters increased in HyperB and decreased in control group after the LPS challenge ($P<0.05$; Table 6). Intramammary LPS challenge decreased mRNA abundance of BDH2, FASN, and OXCT1 in LPS quarters of both treatment groups, and decreased BDH1 and GLUT4 mRNA abundance in control group ($P<0.05$). Infusion of BHBA decreased BDH2 mRNA abundance in control quarters in HyperB group ($P<0.05$) after the LPS challenge. The mRNA abundance of other candidate genes in control quarters were not affected by LPS challenge and BHBA infusion in both treatment groups.

DISCUSSION

To our best knowledge, this experiment is the first study to investigate effects of an induced long term (56 h) hyperketonemia, without other metabolic effects of NEB, on mammary gland metabolism, metabolic and endocrine parameters in combination with an activation of immune response by an intramammary LPS administration in mid lactating dairy cows.

The effects of induced hyperketonemia for 48 h on blood metabolites, feed intake, mRNA abundance of candidate genes related to hepatic metabolism, and milk yield have been reported earlier (Zarrin et al., 2013a). The moderate effects of BHBA infusion on mammary gland metabolism at mRNA level confirms that milk synthesis was not affected by intravenous BHBA infusion in mid lactating dairy cows (Zarrin et al., 2013a). The lacking effect on milk synthesis was likely due to the related low needs of glucose for mammary lactose synthesis and other metabolic processes at this lactational stage compared to early lactation. Thus the reduced plasma glucose levels during BHBA infusion were likely not limiting for milk secretion.

Intramammary LPS challenge decreased plasma BHBA in both treatment groups. The goal to maintain plasma BHBA at 1.7 mmol/L through an increased BHBA infusion rate could not be achieved because the decline of plasma BHBA was faster than the adjustment of infusion rate was possible. Waldron et al. (2003) suggested that the decline of plasma BHBA concentration was related to the suppression of ruminal epithelium ketogenesis capacity after LPS challenge. Because the decline of BHBA after LPS administration was quite rapid and pronounced a contribution of changed ruminal absorption may be excluded. Despite not doing a mammary LPS challenge, intra-muscularly applied LPS in a different species decreased hepatic ketogenesis capacity (Memon et al., 1992) while LPS administration did not affect hepatic ketogenesis capacity in incubated liver slices of dairy cows (Waldron et al., 2003). Based on the fast and huge changes of plasma BHBA in BHBA infused animals in the present study this effect seems very unlikely. Recent findings showed that LPS challenge increased milk BHBA concentration in LPS treated quarters during an induced hyperketonemia (Lehmann et al., 2013). Thus a certain quantity of BHBA is lost with the secreted milk in LPS stimulated quarters. However, this portion does not seem to be quantitatively of great importance because milk secretion is reduced in the quarters treated with LPS.

A transient increase and a subsequent decrease of plasma glucose concentration after LPS challenge in both groups in this study was consistent with previous studies that illustrated a transient hyperglycemia after *E. coli* endotoxin induced mastitis (Bruckmaier et al., 1993), increased and subsequent decreased plasma glucose concentration after LPS challenge (Werling et al., 1996). Hypoglycemia after the LPS challenge can also be attributed to increases of cytokines such as TNFα and IL-1β (Schmitz et al., 2004; Vernay et al., 2012; Zarrin et al., 2013b) that increase glucose utilization during the LPS challenge (Stouthard et al., 1995). Regarding to increase plasma glucose concentration after the LPS challenge, during a similar immune stimulation via LPS challenge previously, development of an insulin

337 resistance was reported, and in this case glucose infusion rate had to be reduced to avoid an
338 increase of plasma glucose concentration, thus additional glucose was available through either
339 glycogenolysis or gluconeogenesis (Vernay et al., 2012). Based on present data it seems that
340 the initial increase plasma glucose concentration after LPS challenge is not related to the
341 glycogenolysis, because LPS challenge increased plasma glucagon concentration 150 min
342 after the LPS administration. The difference between plasma glucose concentrations in the
343 two experimental groups after the LPS challenge is related to the BHBA infusion that
344 decreased glucose production besides the decreased gluconeogenesis during LPS. The low
345 plasma glucose concentration in HyperB rather than in the control group may be related to the
346 reduced increase of plasma glucagon concentration in HyperB compared to the control group
347 which resulted in low glucose production from gluconeogenesis or glycogen storage (Zarrin et
348 al., 2013a).

349 Intramammary LPS challenge did not affect plasma NEFA concentration in the present study.
350 This finding is in agreement with a previous study that showed intramammary LPS challenge
351 did not affect plasma NEFA concentration, whereas plasma NEFA concentration increased in
352 control cows that received NaCl (Waldron et al., 2006). The results of the present study are in
353 contrast with a previous study where different doses of LPS were administrated intravenously
354 for 100 min in midlactating dairy cows and plasma NEFA tended to increase after LPS
355 challenge (Waldron et al., 2003). In our previous study intramammary LPS challenge caused
356 a decrease of plasma NEFA concentration in mid lactating dairy cows during hypoglycemic
357 and euglycemic clamps which manipulated plasma glucose concentration (Vernay et al.,
358 2012). It can be assumed that in the present study the unaffected plasma NEFA concentration
359 following LPS challenge is related to the use of BHBA as an alternative energy source which
360 can diminish mobilization of fatty acids from fat stores in the HyperB group.

As previously observed in response to intramammary LPS administration (Lehtolainen et al., 2003; Waldron et al., 2006; Vernay et al., 2012), plasma cortisol concentration increased in both treatment groups. Pro-inflammatory cytokines and possibly also the handling of the animals during the experiments likely activated the hypothalamus-pituitary-gland axis and increased the synthesis of glucocorticoids (Beishuizen and Thijs, 2003). Elevation of plasma cortisol concentration may be partially responsible for the observed induction of an insulin resistance (Andrews and Walker, 1999) to provide more glucose for the immune reaction.

The increase of glucagon after LPS administration is in agreement with Waldron et al. (2003) who reported plasma glucagon concentration being increased at 2 h after the LPS challenge and stayed at a high level up to 8 h after the start of LPS challenge. In the current study increased plasma glucagon concentration in both groups was observed at 150 min after the intramammary LPS administration. However, the increase of plasma glucagon concentration during LPS challenge was much less in cows that received BHBA compared to Control. As reported previously, BHBA infusion decreased glucagon secretion (Zarrin et al., 2013a), that was probably mediated by the inhibitory effect of gamma amino butyric acid (GABA) on glucagon secretion (Adeghate et al., 2000; Wendt et al., 2004), which increased in the epileptic brain in present of high plasma BHBA concentration (Suzuki et al., 2009). The less pronounced increase of glucagon during LPS challenge in the HyperB group is likely related to a lower need of glucose for the immune response than in the control group, and thus less activation of gluconeogenesis. This effect is another indicator for BHBA to be an alternative energy source.

An increased plasma insulin concentration in response to the LPS challenge in the present study is in agreement with previous studies (Waldron et al., 2003; Waldron et al., 2006; Vernay et al., 2012). Despite the inhibitory effect of insulin on glucagon secretion (Weir et al., 1976), plasma glucagon concentration increased after LPS challenge. The effect of LPS

challenge on glucoregulatory hormones is most likely related to effects of pro inflammatory cytokines (Eizirik et al., 1995; Andersson et al., 2001) which are stimulating the pancreatic production and release of these hormones.

The effect of the intramammary LPS challenge on FASN, OXCT1, and BDH2 mRNA abundance in quarters stimulated with LPS in both groups, and decrease of CS mRNA abundance in the control group showed that LPS challenge negatively affects mammary gland metabolism and milk synthesis, which had been reported before (Waldron et al., 2003; Waldron et al., 2006). The increase of CS mRNA abundance in HyperB after LPS challenge is in agreement with Bionaz and Loor (2008) who suggested that the major product of BHBA metabolism in the bovine mammary gland is citrate which is increased in milk. It can be assumed that the upregulating of mammary gland CS mRNA abundance in HyperB group confirmed that BHBA can be partly used to produce energy through the tricarboxylic acid cycle by mammary gland (Palmquist et al., 1969).

CONCLUSIONS

Results demonstrate that BHBA represents an alternative energy source for the mammary tissues and for the immune system. With respect to milk secretion and the related specific need of glucose the BHBA induced adaptions may cause an inadequate decline of plasma glucose. It can be speculated that the resulting low plasma glucose concentration does also negatively affect the immune response. The current results may reflect the negative effect of spontaneous hyperketonemia on hepatic gluconeogenesis by providing less glucose. A long-term reduced glucose availability caused by hyperketonemia might lower the productive performance of dairy cows.

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Figures legends

Figure 1. Mean beta-hydroxybutyrate (BHBA) infusion rate during -24 to -48 h infusion (24 - 48h inf) and hourly after LPS challenge (510 min) in BHBA cows (HyperB, n=5). Mean plasma BHBA concentration in cows infused with BHBA (HyperB) and control cows (NaCl, n=8) during 48 h infusion -24 to -48 h average and hourly after LPS challenge (510 min). Values represent mean ± SEM. The open squares with plus show significant difference (P<0.05) of BHBA infusion rate during the LPS challenge to mean infusion rates in-24 to -48 h. The close circles and open circles show significant differences (P<0.05) between -24 to -48 h average and each time point during the LPS challenge for HyperB, and NaCl group, respectively. The closed rectangle indicates significant difference between two treatment groups during LPS challenge (P<0.05).

Figure 2. Mean plasma glucose (A), glucagon (B), and insulin (C) concentration in cows infused with BHBA (HyperB, n=5) and control cows (NaCl, n=8) during 48 h infusion (-24 to -48 h average) and hourly after LPS challenge (510 min). Values represent mean ± SEM. The close circles and open circles show significant differences (P<0.05) between -24 to -48 h average and each time point during the LPS challenge for HyperB, and NaCl group, respectively. The closed rectangle indicates significant difference between two treatment groups during LPS challenge (P<0.05).

Figure 3. Mean plasma NEFA (A), urea (B), and cortisol (C) concentration in cows infused with BHBA (HyperB, n=5) and control cows (NaCl, n=8) during 48 h infusion (-24 to -48 h average) and hourly after LPS challenge (510 min). Values represent mean ± SEM. The close circles and open circles show significant differences (P<0.05) between -24 to -48 h average and each time point during the LPS challenge for HyperB, and NaCl group, respectively. The closed rectangle indicates significant difference between two treatment groups during LPS challenge (P<0.05).

Figure 4. Insulin: glucagon molar ratio in cows infused with BHBA (HyperB, n=5) and control cows (NaCl, n=8) during 48 h infusion (-24 to -48 h average) and hourly after LPS challenge (510 min). Values represent mean ± SEM. The close circles and open circles show significant differences (P<0.05) between -24 to -48 h average and each time point during the LPS challenge for HyperB,

and NaCl group, respectively. The closed rectangle indicates significant difference between two treatment groups during LPS challenge (P<0.05).

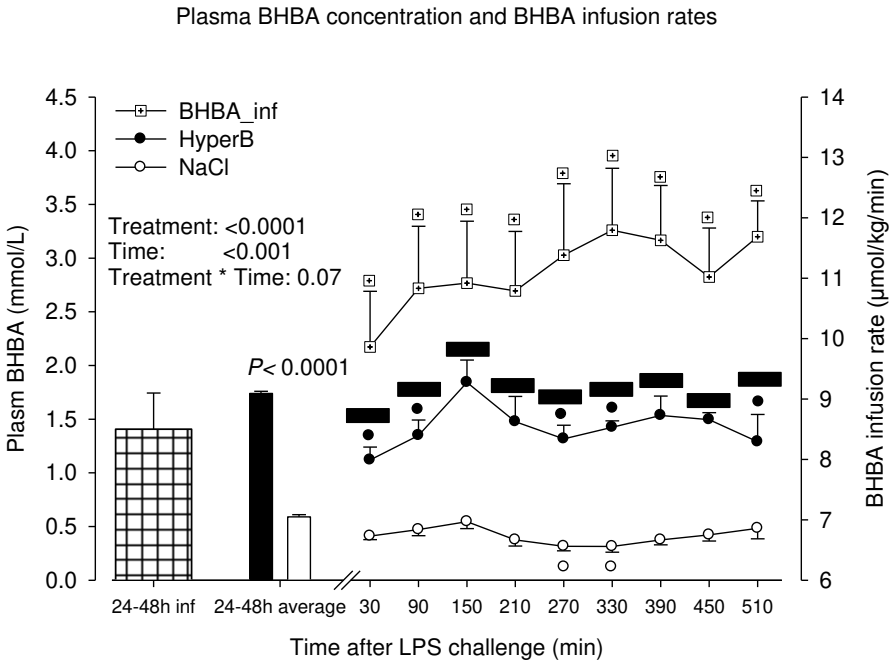
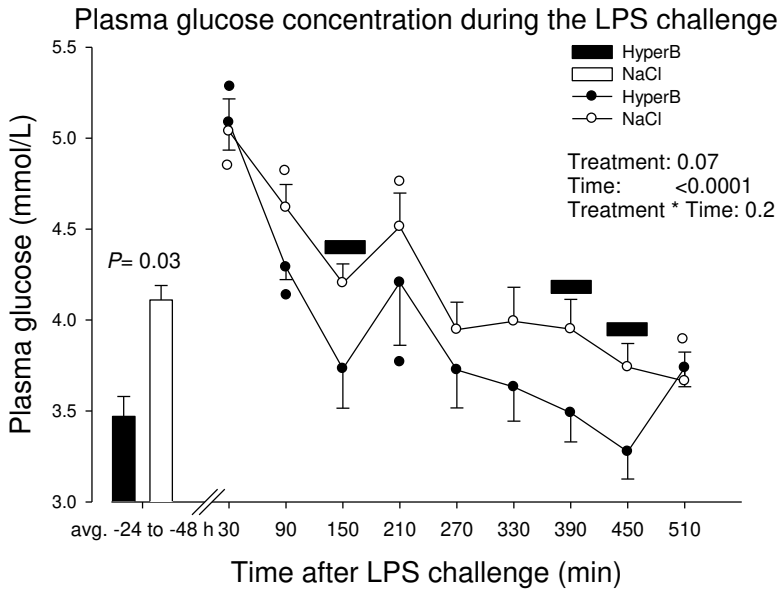


Figure 2A



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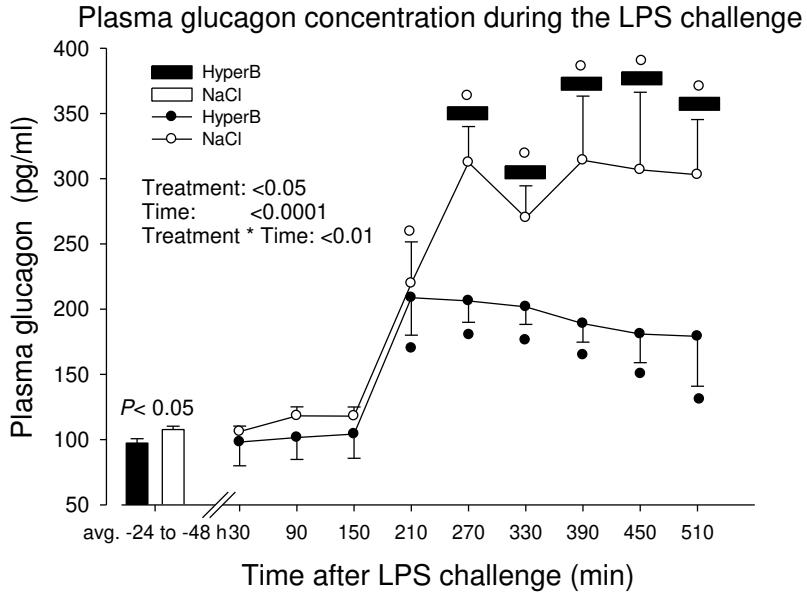

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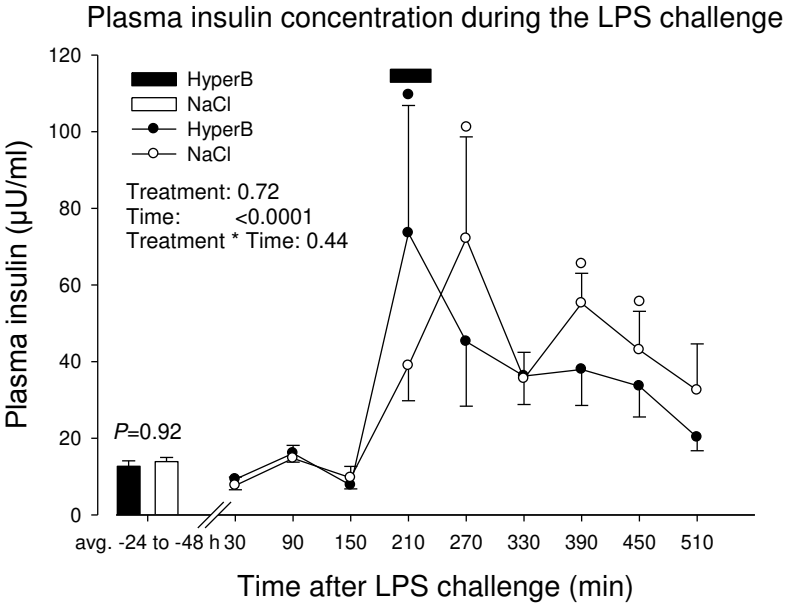


Figure 3A

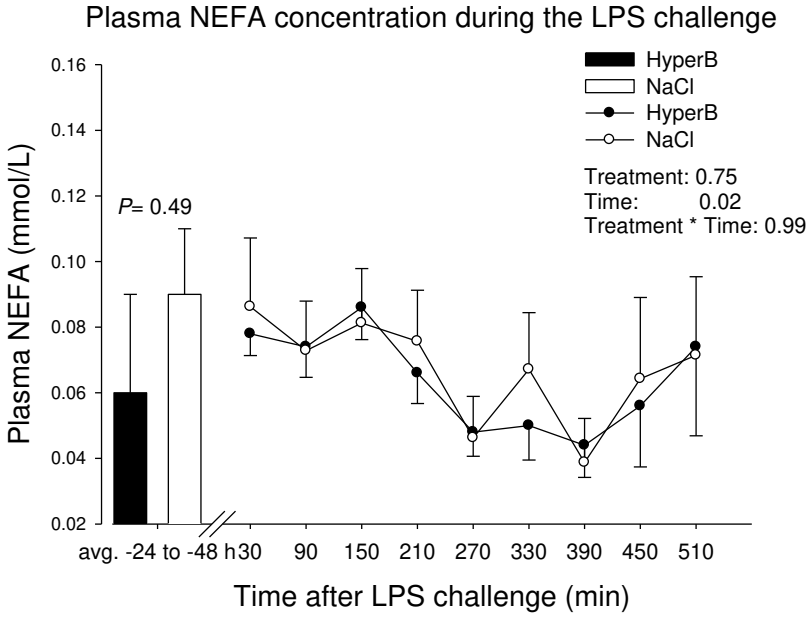
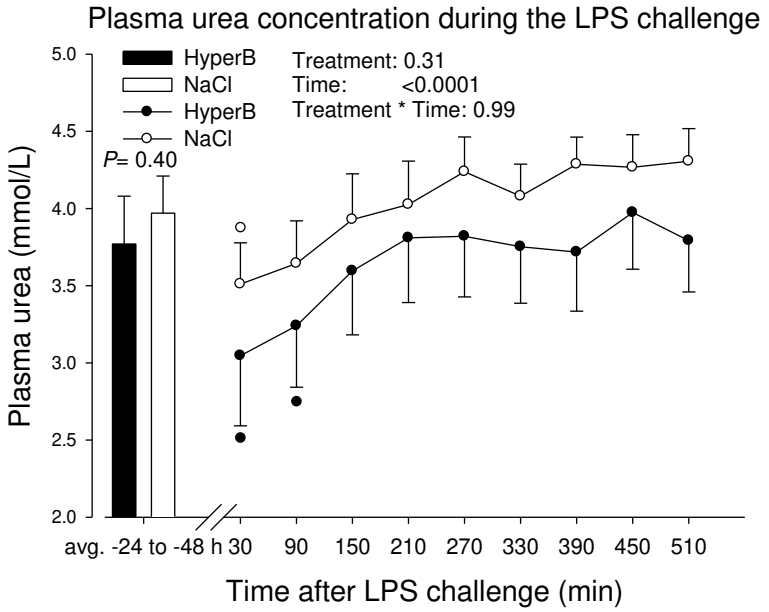


Figure 3B



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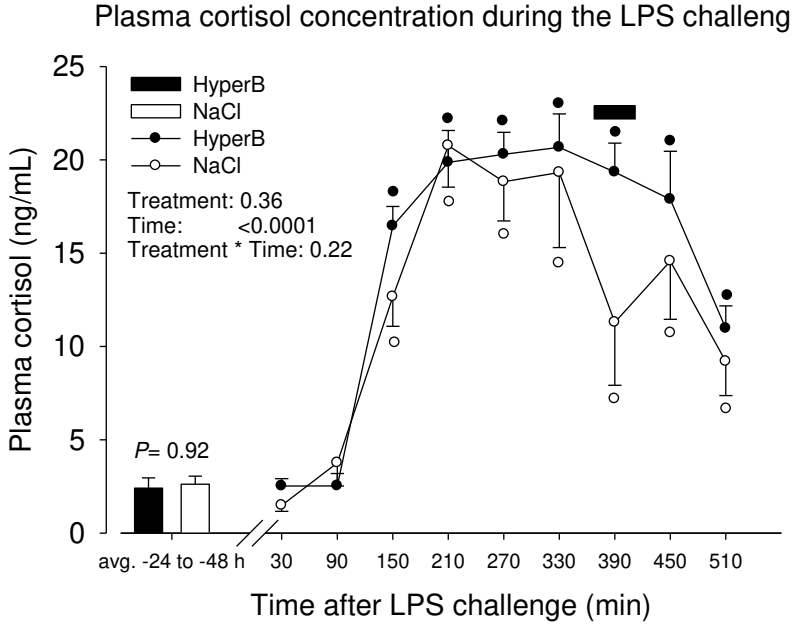

Figure 4

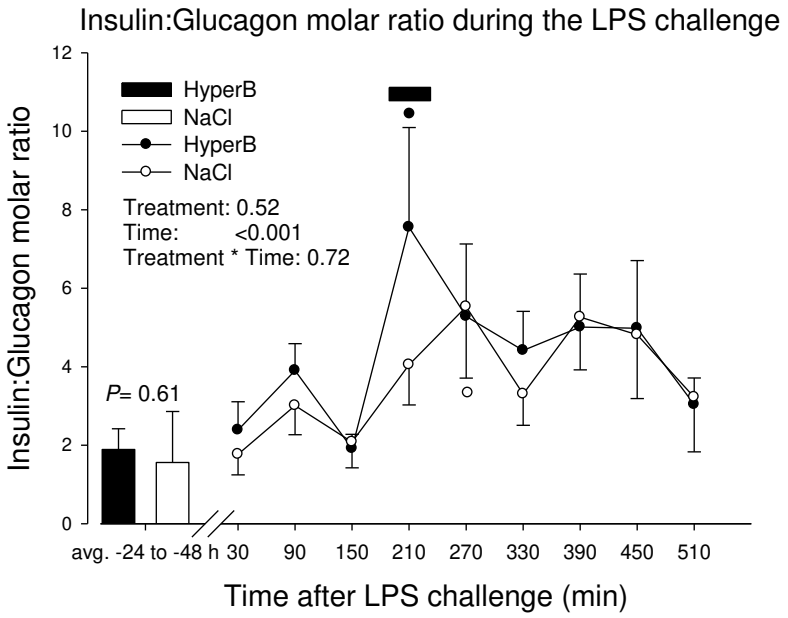
Insulin:Glucagon molar ratio during the LPS challenge
HyperB
NaCl
HyperB
NaCl
Treatment: 0.52
Time: <0.001
Treatment * Time: 0.72
P= 0.61
Insulin:Glucagon molar ratio
0
2
4
6
8
10
12
avg. -24 to -48 h
30
90
150
210
270
330
390
450
510
Time after LPS challenge (min)

Table 1. Polymerase chain reaction primer information (for = forward, rev = reverse), annealing temperature and the PCR product length

Gene[1]		Sequence 5'-3'	Gene Bank accession no.	Annealing temperature (°C)	Length (bp)
Fatty acid synthesis related variables					
ACoAC	for	CTCTTCCGACAGGTTCAAGC	AJ-132890	61	248
	rev	ACCATCCTGGCAAGTTTCAC			
FASN	for	CTGAGTCGGAGAACCTGGAG	NM_001012669	63	232
	rev	ACAATGGCCTCGTAGGTGAC			
Beta-hydroxybutyrate metabolism related variables					
BDH1	for	GAGTGAGAGCGGGTAAGGGT	NM_001034600.2	61	199
	rev	GGCCACAAAAGGCAGAATGG			
BDH2	for	TGCAACTGTGTGTGTCCAG	NM_001034488.2	58	175
	rev	CAGATTCATCAGAGGCCAAG			
OXCT1	for	ATGGTGACCTGGCTAACTGG	NM_001076070.2	60	234
	rev	TCAGACCCTTTTTGCTGTCC			
Citrate synthase					
CS	for	TGGACATGATGTATGGTGG	BC-114138	60	217
	rev	AGCCAAGATACCTGTTCCTC			
Glucose transporters					
GLUT1	for	GCTTCTCCAACTGGACTTCG	NM_174602	60	225
	rev	ACAGCTCCTCAGGTGTCTTG			
GLUT4	for	GACTGGTACCCATGTACGTG	NM_174604.1	60	242
	rev	CCGGATGATGTAGAGGTAGC			
Housekeeping genes					
GAPDH	for	GTC TTC ACT ACC ATG GAG AAG G	NM001034034	60	197
	rev	TCA TGG ATG ACC TTG GCC AG			
Ubiquitin	for	AGA TCC AGG ATA AGG AAG GCA T	NM174133	62	198
	rev	GCT CCA CCT CCA GGG TGA T			

[1]ACoAC = acetyl-CoA carboxylase; FASN = fatty acid synthase; BDH1 = beta-hydroxybutyrate dehydrogenase 1; BDH2 = beta-hydroxybutyrate dehydrogenase 2; OXCT1 = succinyl-CoA:3-ketoacid-coenzyme A transferase 1; CS = citrate synthase; GLUT1 = glucose transporter 1; GLUT4 = glucose transporter 4; GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

Table 2. Means ± SEM of mRNA levels of housekeeping genes across time points and treatments.

Genes[1]	Treatment[2]	Baseline	48h after infusions	End of infusions
GAPDH	HyperB	18.6 ± 0.3	17.9 ± 0.2	17.3 ± 0.1
	NaCl	19.9 ± 0.7	19.3 ± 0.7	17.8 ± 0.7
Ubiquitin	HyperB	16.8 ± 0.4	17.2 ± 0.7	16.6 ± 0.2
	NaCl	19.1 ± 0.4	18.5 ± 0.3	18.1 ± 0.5

[1]GAPDH = glyceraldehyde 3-phosphate dehydrogenase.
[2]HyperB = Hyper beta-hydroxybutyrate group (n=5); NaCl = group of cows receiving physiological saline solution (n=8).

Table 3. Milk yield, DMI of dairy cows infused with beta-hydroxybutyrate (HyperB, n=5) or saline (NaCl, n=8) before the start of the infusion (Day 0), second day of infusion (Day 2), and third day of infusion (day of LPS challenge). Values represent Mean ± SEM.

Variables[1]	Treatment[2]	Day 0	Day 2	Day 3 (LPS challenge)	P-Value, Treatment	P-Value, Time	P-Value, Treatment* Time
Milk yield, kg/d	HyperB	24.9 ± 2.6	22.0 ± 2.6	13.0 ± 2.6	0.94	<0.01	0.43
	NaCl	23.0 ± 2.3	20.6 ± 2.0	16.7 ± 2.0			
DMI, kg/d	HyperB	19.9 ± 1.9	19.0 ± 1.9	8.5 ± 1.9	0.41	<0.001	0.17
	NaCl	19.4 ± 1.7	18.1 ± 1.5	13.5 ± 1.5			

[1]DMI= dry matter intake.
[2]HyperB = Hyper beta-hydroxybutyrate group (n=5); NaCl = group of cows receiving physiological saline solution (n=8).

Table 4. Plasma variables concentration in dairy cows infused with beta-hydroxybutyrate (HyperB, n=5) or saline (NaCl, n=8) during the LPS challenge. Data are presented as least square means (LSM) ± SEM of area under the curve (AUC) during 8 h. AUC was calculated according the Trapezoidal rule.

Variable [1]	NaCl	HyperB	*P*-Value, NaCl vs. Baseline	*P*-Value, HyperB vs. Baseline	*P*-Value, HyperB vs. NaCl
	LSM ± SEM	LSM ± SEM			
Glucose, mmol/L*h	4.3 ± 0.1	3.8 ± 0.1	0.01	0.30	0.02
BHBA, mmol/L*h	0.4 ± 0.0	1.4 ± 0.1	0.03	<0.001	< 0.001
NEFA, mmol/L*h	0.1 ± 0.0	0.1 ± 0.0	0.81	0.20	0.76
Urea, mmol/L*h	4.0 ± 0.3	3.7 ± 0.3	0.02	0.07	0.44
Cortisol, ng/mL*h	13.8 ± 1.5	14.7 ± 2.0	<0.001	0.001	0.73
Insulin, mU/L*h	35.0 ± 5.4	34.8 ± 6.9	<0.001	0.01	0.97
Glucagon, pg/ml*h	221.7 ± 15.3	161.2 ± 19.3	<0.001	0.08	0.03
Insulin: Glucagon, molar ratio*h	3.7 ± 0.7	4.4 ± 0.9	0.03	0.02	0.52

[1]BHBA = beta-hydroxybutyrate; NEFA = non-esterified fatty acids.

[2]HyperB = Hyper beta-hydroxybutyrate group (n=5); NaCl = group of cows receiving physiological saline solution (n=8).

Table 5. Changes of mRNA abundance of genes related to mammary gland metabolism during 48 h infusion with beta-hydroxybutyrate (HyperB) or saline (NaCl). Delta (differences between before and 48 h after the start of infusions). Values represent Mean ± SEM.

Parameter	Group	Delta	ANOVA (P-Value,group)
Fatty acid synthesis related variables			
ACoAC	HyperB	-1.0 ± 0.5	0.16
	NaCl	0.0 ± 0.4	
FASN	HyperB	-1.0 ± 0.3*	0.13
	NaCl	-0.2 ± 0.5	
Beta-hydroxybutyrate metabolism related variables			
BDH1	HyperB	-0.9 ± 0.5	0.49
	NaCl	-0.2 ± 0.6	
BDH2	HyperB	0.1 ± 0.4	0.53
	NaCl	0.6 ± 0.6	
OXCT1	HyperB	-0.5 ± 0.3	0.66
	NaCl	-0.2 ± 0.4	
Citrate synthase			
CS	HyperB	-0.2 ± 0.3	0.86
	NaCl	-0.1 ± 0.4	
Glucose transporters			
GLUT1	HyperB	-0.4 ± 0.2	0.88
	NaCl	-0.5 ± 0.7	
GLUT4	HyperB	0.6 ± 0.5	0.14
	NaCl	-0.4 ± 0.4	

[1]ACoAC = acetyl-CoA carboxylase; FASN = fatty acid synthase; BDH1 = beta-hydroxybutyrate dehydrogenase 1; BDH2 = beta-hydroxybutyrate dehydrogenase 2; OXCT1 = succinyl-CoA:3-ketoacid-coenzyme A transferase 1; CS = citrate synthase; GLUT1 = glucose transporter 1; GLUT4 = glucose transporter 4.
[2]HyperB = Hyper beta-hydroxybutyrate group (n=5); NaCl = group of cows receiving physiological saline solution (n=8).
*Delta is different from 0 (P<0.05).

Table 6. Differences in mRNA abundance related to metabolism in mammary gland during the LPS challenge in LPS and control quarters in dairy cows infused with beta-hydroxybutyrate (HyperB) or saline (NaCl). Values are represented as mean ± SEM.

Parameter	Group	LPS Delta (after LPS – before LPS)	ANOVA (*P*-Value,group)	Control Delta (after LPS – before LPS)	ANOVA (*P*-Value,group)
Fatty acid synthesis related variables					
ACoAC	HyperB	-0.9 ± 0.7	0.66	-0.6 ± 0.5	0.58
	NaCl	-0.5 ± 0.6		-1.1 ± 0.7	
FASN	HyperB	-4.4 ± 0.7*	0.60	-1.0 ± 1.2	0.47
	NaCl	-3.8 ± 0.8*		-0.2 ± 0.5	
Beta-hydroxybutyrate metabolism related variables					
BDH1	HyperB	-1.4 ± 0.9	0.37	-1.6 ± 1.1	0.96
	NaCl	-2.7 ± 0.9*		-1.7 ± 0.8	
BDH2	HyperB	-2.6 ± 0.4*	0.87	-1.3 ± 0.5*	0.62
	NaCl	-2.4 ± 0.8*		-1.0 ± 0.5	
OXCT1	HyperB	-1.8 ± 0.3*	0.25	0.3 ± 0.7	0.56
	NaCl	-1.0 ± 0.5*		1.7 ± 1.8	
Citrate synthase					
CS	HyperB	0.5 ± 0.3	0.05	-0.4 ± 0.3	0.35
	NaCl	-0.5 ± 0.3		0.3 ± 0.5	
Glucose transporters					
GLUT1	HyperB	-0.4 ± 0.4	0.74	0.4 ± 0.4	0.85
	NaCl	-0.5 ± 0.2		0.3 ± 0.5	
GLUT4	HyperB	-0.9 ± 0.9	0.24	0.8 ± 0.8	0.24
	NaCl	-2.0 ± 0.5*		0.4 ± 0.6	

[1]ACoAC = acetyl-CoA carboxylase; FASN = fatty acid synthase; BDH1 = beta-hydroxybutyrate dehydrogenase 1; BDH2 = beta-hydroxybutyrate dehydrogenase 2; OXCT1 = succinyl-CoA:3-ketoacid-coenzyme A transferase 1; CS = citrate synthase; GLUT1 = glucose transporter 1; GLUT4 = glucose transporter 4.

[2]HyperB = Hyper beta-hydroxybutyrate group (n=5); NaCl = group of cows receiving physiological saline solution (n=8).

*Delta is different from 0 ($P<0.05$).